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Vacuolar sequestration of glutathione *S*-conjugates outcompetes a possible degradation of the glutathione moiety by phytochelatin synthase

Anke Grzam[a], Pierre Tennstedt[b], Stephan Clemens[b], Rüdiger Hell[a], Andreas J. Meyer[a,*]

[a] *Heidelberg Institute of Plant Sciences, University of Heidelberg, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany*
[b] *Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany*

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Abstract **Monochlorobimane was used as a model xenobiotic for Arabidopsis to directly monitor the compartmentation of glutathione-bimane conjugates in situ and to quantify degradation intermediates in vitro. Vacuolar sequestration of the conjugate was very fast and outcompeted carboxypeptidation to the γ-glutamylcysteine-bimane intermediate (γ-EC-B) by phytochelatin synthase (PCS) in the cytosol. Following vacuolar sequestration, degradation proceeded to cysteine-bimane without intermediate. Only co-infiltration of monochlorobimane with Cd^{2+} and Cu^{2+} increased γ-EC-B formation to 4% and 25%, respectively, within 60 min. The role of PCS under simultaneous heavy metal stress was confirmed by investigation of different *pcs1* null-mutants. In the absence of elevated heavy metal concentrations glutathione-conjugates are therefore first sequestered to the vacuole and subsequently degraded with the initial breakdown step being rate-limiting.**

Keywords: Glutathione-conjugate degradation; Monochlorobimane; Phytochelatin synthase; Vacuolar sequestration

1. Introduction

Degradation and sequestration of noxious compounds, whether generated endogenously through side reactions of normal metabolism or supplied from outside, is essential for the survival of plants. Many electrophilic compounds including several economically important herbicides are detoxified through the GSH-based detoxification system in which the toxic compounds are conjugated to reduced glutathione (GSH) via the nucleophilic thiol residue. The conjugation reaction between GSH and electrophiles is generally considered to be catalysed by cytosolic glutathione *S*-transferases [1]. The GSH-moiety of the conjugate then acts as a tag identifying the toxic compound for vacuolar sequestration through action of transporters belonging to the multidrug resistance-associated (MRP) family of a large and diverse group of ABC-transporters [2,3].

In both animals and plants glutathione conjugates have been shown to be degraded, but up to now the entire degradation pathway has only been fully elucidated in animals. In the mammalian degradation pathway the hydrolysis of the γ-glutamyl residue by γ-glutamyl-transferases (GGTs) is considered to be the initial degradation step, followed by carboxypeptidation of the glycine residue [4]. In plants, however, the conjugates are generally assumed to be transported into the vacuole where they are converted through a concerted action of different enzymes to the respective cysteine conjugate. It has been shown that plant GGTs can catalyze cleavage of GSH-conjugates to release the γ-glutamyl-residue [5]. Preliminary evidence for the involvement of a vacuolar GGT in degradation of GSH-conjugates in radish (*Raphanus sativum* L.) vacuoles was provided by Nakano and Sekiya [6]. In this case cleavage of the γ-glutamyl residue has been reported as the initial step of degradation. In contrast, Wolf et al. [7] described the activity of a vacuolar carboxypeptidase cleaving the glycine residue as the initial step of degradation in barley resulting in accumulation of the intermediate γ-EC. More recently, a carboxypeptidase activity towards GSH-conjugates as the initial degradation step was also shown for phytochelatin synthase (PCS) from *Silene cucubalus* and *Arabidopsis thaliana* [8]. PCS has been found in plants, many fungi and some animals. It is constitutively expressed, but forms $(\gamma\text{-EC})_n$G (phytochelatins) from GSH only in the presence of heavy metals [9]. In vitro PCS can effectively act on GSH-conjugates as a carboxypeptidase, cleaving the glycine residue during transfer of the γ-glutamylcysteinyl conjugate to a water molecule [8]. Given that PCS is a cytosolic enzyme this observation would imply at least partial degradation of the conjugates in the cytosol.

The GSH-based detoxification pathway can be traced with the fluorescent dye monochlorobimane (MCB) allowing for in situ observations of the initial detoxification processes [10]. Labelling of Arabidopsis cells with MCB is specific for GSH because this reaction is catalysed by glutathione *S*-transferases (GSTs) [10,11]. Absence of detectable MCB-labelling in GSH-deficient mutants further corroborated the high specificity of the labelling [12]. After conjugation of GSH with MCB

*Corresponding author. Fax: +49 6221 54 5859.
E-mail address: ameyer@hip.uni-hd.de (A.J. Meyer).

Abbreviations: Cys-B, cysteine-bimane; CysGly-B, cysteinylglycine-bimane; γ-EC-B, γ-glutamylcysteine-bimane; GGT, γ-glutamyl-transferase; GSB, glutathione *S*-bimane; GSH, reduced glutathione; GST, glutathione *S*-transferase; MBB, monobromobimane; MCB, monochlorobimane; LMWT, low molecular weight thiol; PCS, phytochelatin synthase; PI, propidium iodide; RT, room temperature

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the vacuolar sequestration of the conjugates can be followed in situ and time course measurements can even provide apparent kinetic parameters for the sequestration process [10,13].

We addressed the cellular organization and sequence of events of the different possible pathways of elimination of the potentially still toxic GSH-conjugates in *A. thaliana* plants. In vitro and in situ data showed that heavy metal activated PCS1 can potentially act as a carboxypeptidase on Glutathione *S*-bimane (GSB). Using time course measurements of fluorescent GSH-conjugates and *pcs1* mutants we show that PCS1, despite a constituting residual carboxypeptidase activity in the absence of high amounts of heavy metals, does not contribute significantly to the metabolism of GSH-conjugates because this degradation is outcompeted by vacuolar sequestration.

2. Materials and methods

2.1. Plant material

A. thaliana (L.) Heynh. (accession Columbia, Col-0) and two mutant lines, deficient in *AtPCS1* were used. Seeds of line *cad1-3* [14] were kindly provided by Chris Cobbett. A T-DNA insertion mutant designated *cad1-6* (Tennstedt et al., unpublished) was isolated from the Garlic collection [15]. All plants were sown on soil and grown for 6–8 weeks at 21 °C under short day conditions (9 h light; 15 h dark) in a controlled growth chamber.

2.2. Leaf infiltration for labelling of GSH

Arabidopsis leaves were cut into small pieces (2 × 2 mm) and vacuum infiltrated for 15 min with buffer (25 mM H_2PO_4/K_2HPO_4, pH 5.2) containing 500 μM MCB (Calbiochem), and the given different divalent cations (100 μM), or NaN_3 (1 mM). For imaging experiments 50 μM propidium iodide (PI) was added to the infiltration solution to label cell walls and prove cell viability. Infiltrated leaf pieces were washed twice with fresh incubation buffer without MCB and metal ions. Leaf pieces were further incubated in buffer at 21 °C in the light for the indicated time. For harvesting, leaf pieces were surface dabbed with absorbent paper, shock frozen in liquid nitrogen, and ground. Fifty milligram of leaf material was extracted with 300 μl 0.1 N HCl. After the second centrifugation (10 min, 14T rpm, 4 °C), the supernatant was appropriately diluted for direct reverse-phase HPLC analysis. For in situ experiments, the amounts of GSB and its degradation products are presented as % of all recovered bimane labelled low molecular weight thiol (LMWT). For quantification, standard solutions with 10–100 μM of each of the four LMWT and extracts with or without prior MCB treatment were derivatized and further analyzed by HPLC as described earlier [16].

2.3. Confocal laser scanning microscopy

Imaging of infiltrated leaf pieces was done and pictures were processed and analyzed as described in Cairns et al. [12] with slight modifications. Images were obtained using a 63× water immersion lens and were collected in line scan mode with an averaging of 4. Noise was further reduced through application of median filters.

2.4. Cell free protein extracts

Leaf material (400–500 mg) of 6–8 weeks old plants were extracted with 800 μl of extraction buffer (100 mM Tris/HCl, pH 8.3; 10% glycerol; 1 mM DTT). After centrifugation (10 min, 14T rpm, and 4 °C) the supernatant was applied to a NAP5 column to separate proteins from low molecular weight compounds. Extraction buffer was used as equilibration and elution buffer. Protein concentrations were determined as described earlier [16].

2.5. Degradation of GSB in cell free extracts

The substrate GSB for in vitro degradation experiments was prepared from GSH and MBB (Calbiochem). For 100 μl of a 5 mM GSB solution, 5 μl 100 mM GSH, 8 μl 100 mM DTT, 10 μl 1 M Tris/HCl, pH 8.3; 5 μl 0.08 N NaOH in a final volume of 71 μl were incubated for 60 min at room temperature (RT). Conjugation was achieved by addition of 26 μl 100 mM monobromobimane (MBB) and incubation for 15 min in the dark at RT. Remaining free MBB was subsequently conjugated to DTT by adding 2 μl 1 M DTT for 15 min, followed by addition of 1 μl of HCl (37%). Purity of the substrate was always confirmed by HPLC.

To assay the GSB degradation activity, 1 mM GSB and 30 μM $CdCl_2$ or $MgCl_2$, respectively, were added to desalted leaf extracts and incubated at RT in the dark for 60 min or as indicated. Samples were taken and added to acetic acid (5%) to terminate the reaction. After centrifugation, the deproteinized supernatants were properly diluted and the LMWT bimane conjugates were analysed by HPLC. To standardize the amount of formed product, results for kinetic measurements were given as pmol bimane–LMWT formed by 1 μg protein and as specific activity of the cell free, desalted leaf extract in $\mu U * mg^{-1}$ protein (U = international unit).

3. Results

3.1. Degradation of GSB in situ

We have used in situ labelling of cells with MCB [10] to investigate the compartmentation of GSH-conjugate degradation and the possible contribution of PCS [8]. Infiltration of *A. thaliana* leaf tissue with 500 μM MCB for 15 min resulted in conjugation of approx. 50% of the total GSH pool and subsequent degradation of the conjugate. Within 48 h after infiltration almost all GSB was degraded through hydrolysis of the two terminal amino acids of the glutathione moiety and a concomitant accumulation of cysteine-bimane (Cys-B) (Fig. 1). The two possible intermediates of this degradation pathway, γ-EC-B and cysteinylglycine-bimane (CysGly-B), were present only in minute amounts during the time course without any sign for long-term accumulation (data not shown). Because the degree of infiltration was variable in different experiments, all data for the different bimane-labelled thiols are presented as the percentage of labelled compounds relative to the sum of labelled LMWTs (180–220 pmol GSB $(mg\ FW)^{-1}$) at the end of the infiltration period. The time course for the degradation of GSB and the accumulation of Cys-B appeared to be biphasic with a plateau between 2 and 4 h after infiltration and a second almost linear phase of accelerated GSB degradation between 4 and 48 h after infiltration (Fig. 1).

The contribution of alternative degradation pathways in both vacuole and cytosol were investigated in more detail for

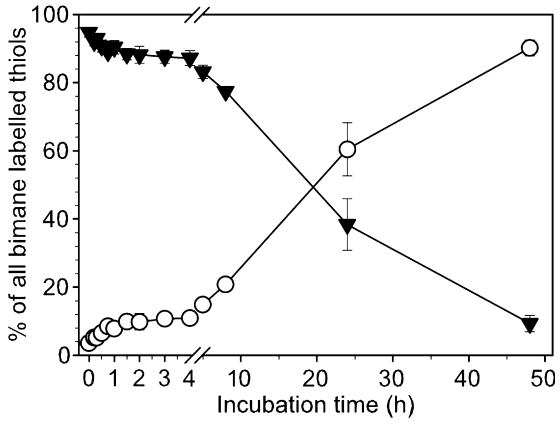

Fig. 1. Intact Arabidopsis cells metabolize GSB to Cys-B. Leaf pieces were infiltrated with 500 μM MCB for 15 min and extracted. Data for GSB (▼) and Cys-B (○) are shown for an incubation period of 48 h after infiltration with MCB. Values shown are means ± S.D. from 3 to 12 independent measurements.

intact plants. To test the hypothesis of cytosolic degradation, we inhibited ATP-synthesis through the infiltration of leaf discs with sodium azide simultaneously with MCB and thus prevented indirectly also the vacuolar sequestration of conjugates. Confocal imaging of bimane-dependent fluorescence in leaves in the absence of azide showed that after vacuum-infiltration with 500 μM MCB almost the entire label was transferred to the vacuole of both epidermal and mesophyll cells after 30 min (Fig. 2A and B). Simultaneous infiltration with 1 mM sodium azide, however, prevented sequestration of GSB and thus led to intense labelling of the cytosol with negatively contrasted vacuoles (Fig. 2C and D). Co-infiltrated PI, a marker for plasma membrane integrity, confirmed in all experiments that the cells remained intact during the infiltration procedure independent of whether azide was included or not.

The possible degradation of GSB in the cytosol was analyzed using leaf discs that were first infiltrated with MCB in the presence of azide and then extracted and subjected to HPLC analysis. Within the first 60 min after infiltration the relative amount of GSB was slightly reduced from 90% in control leaves labelled in the absence of azide to 83% in leaves labelled with MCB plus azide. The decrease in detectable GSB was accompanied by an increase of the relative amount of Cys-B to 15% in the presence of azide. Only minute relative amounts of γ-EC-B intermediate and CysGly-B of around 1% were detected, and no significant effect of azide on the relative amounts of these compounds was observed (Table 1). Very similar data were measured for the degradation of GSB after infiltration of the PCS1-deficient mutant *cad1-3* with MCB. The amount of γ-EC-B formed within 60 min in this case was even lower than in wild-type. Other inhibitors known to inhibit ABC-transporters on isolated tonoplast vesicles like vanadate caused only partial inhibition of vacuolar sequestration even at millimolar concentration in intact cells (data not shown). Therefore these inhibitors were not suitable for further elucidation of the contribution of cytosolic PCS to GSB degradation.

Table 1
The conversion of GSB in living Arabidopsis cells is not affected by co-infiltration with azide and in the absence of heavy metals independent of phytochelatin synthase

Infiltration medium		Wild-type		*cad1-3*	
		MCB	MCB + Azide	MCB	MCB + Azide
Cys-B	0 min	3.6 ± 0.9	3.9 ± 1.6	2.6 ± 0.4	5.8 ± 0.8
	30 min	6.6 ± 1.1	9.0 ± 3.5	7.4 ± 1.6	9.5 ± 2.8
	60 min	8.0 ± 1.4	15.1 ± 1.9	7.8 ± 2.0	13.8 ± 2.2
γ-EC-B	0 min	1.1 ± 0.8	1.3 ± 1.0	0.4 ± 0.2	0.5 ± 0.1
	30 min	1.8 ± 0.7	1.4 ± 0.6	0.5 ± 0.1	0.5 ± 0.1
	60 min	1.0 ± 0.6	1.4 ± 0.6	0.5 ± 0.1	0.6 ± 0.1
CysGly-B	0 min	0.6 ± 0.2	0.6 ± 0.3	0.5 ± 0.1	0.6 ± 0.1
	30 min	0.9 ± 0.4	1.0 ± 0.3	1.1 ± 0.3	0.8 ± 0.03
	60 min	0.8 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.2
GSB	0 min	94.7 ± 1.4	94.2 ± 1.9	96.6 ± 0.5	93.0 ± 1.0
	30 min	90.7 ± 1.8	88.6 ± 3.0	91.0 ± 1.8	89.2 ± 2.9
	60 min	90.2 ± 1.9	82.6 ± 2.1	90.7 ± 2.1	84.6 ± 2.5

Leaf pieces of wild-type and *cad1-3* leaves were vacuum infiltrated with 500 μM MCB with or without 1 mM Na-azide for 15 min. Samples were taken immediately after the infiltration (0 min) or after an additional incubation for 30 min and 60 min in buffer without MCB. All values are presented as percentage of all bimane labelled LMWT because the degree of labelling can vary from one experiment to another. Values are means ±S.D. of 3–12 experiments.

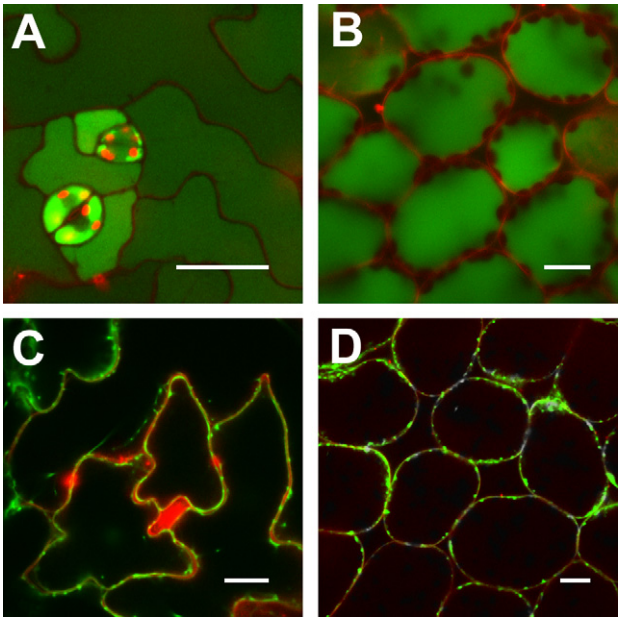

Fig. 2. Co-infiltration with azide inhibits vacuolar sequestration of GSB in Arabidopsis leaves. Leaf pieces were vacuum infiltrated with 500 μM MCB and 50 μM PI in the absence (A and B) or the presence (C and D) of 1 mM Na-azide for 15 min and incubated for additional 30 min. The images show single optical sections through the epidermal cell layer (A and C) and the palisade parenchyma cells (B and D), respectively. Scale bars = 20 μm.

3.2. GSB degradation can be affected by cadmium

PCS is known to be constitutively expressed, but the enzyme is only active in terms of phytochelatin synthesis in the presence of heavy metals [9]. To investigate whether degradation of GSB is influenced by the presence of heavy metals, 1 mM GSB was incubated with cell free extracts in the presence or absence of 30 μM Cd^{2+} under optimal buffer conditions for PCS1 [8]. In the absence of Cd^{2+} all three possible degradation products, γ-EC-B, CysGly-B and Cys-B, were found to accumulate with CysGly-B being present at the highest level. The kinetics, however, were found to be different. Formation for CysGly-B showed a plateau, whereas γ-EC-B and Cys-B showed a slightly accelerated accumulation during the time course of 2 h (Fig. 3). After a 2 h incubation period about 40 pmol (μg protein)$^{-1}$ CysGly-B were formed. Besides this about 15 pmol (μg protein)$^{-1}$ Cys-B and 3 pmol (μg protein)$^{-1}$ γ-EC-B were produced (Fig. 3). The presence of Cd^{2+} had no influence on formation of CysGly-B and Cys-B, but significantly increased the amount of γ-EC-B formed to 8 pmol (μg protein)$^{-1}$. Thus, after 2 h the amount of γ-EC-B was about 2.5 fold higher than in the control, indicating the activation of another degrading enzyme by Cd^{2+} (Fig. 3A).

The identity of cytosolic PCS activity responsible for the observed Cd^{2+}-dependent increase of γ-EC-B formation was verified by using cell free extracts of the PCS1-deficient mutants *cad1-3* and *cad1-6*. Both mutants showed only very little accumulation of γ-EC-B. Even in the absence of Cd^{2+} the amount of γ-EC-B formed from GSB during the 1 h incubation period

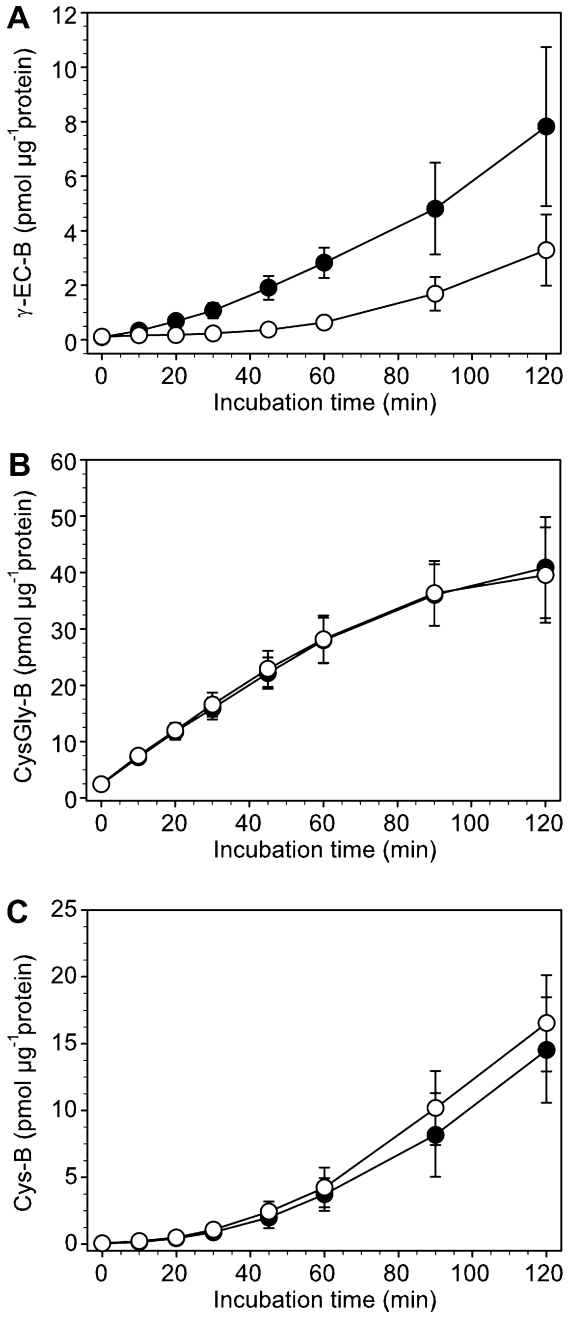

Fig. 3. Cd^{2+} affects the degradation of GSB in cell free extracts. Extracts of WT leaves were incubated with 1 mM GSB in the presence of 30 µM $MgCl_2$ (○) or $CdCl_2$ (●). Data show the formation of γ-EC-B (A), CysGly-B (B) and Cys-B (C) per µg protein. All values are means ±S.D. ($n = 3$).

in extracts from PCS1 mutants was significantly lower than in extracts from wild-type plants (Fig. 4). Arabidopsis contains a second gene, *AtPCS2*, which encodes a second functional PCS [17]. However, knockout of this gene did not result in any changes in degradation of GSB observed in cell free assays (data not shown).

3.3. Activation of PCS1-mediated degradation of GSB by different heavy metals

The possible role of PCS1 in degradation of GSH-conjugates in living cells was investigated in leaf disks of wild-type plants

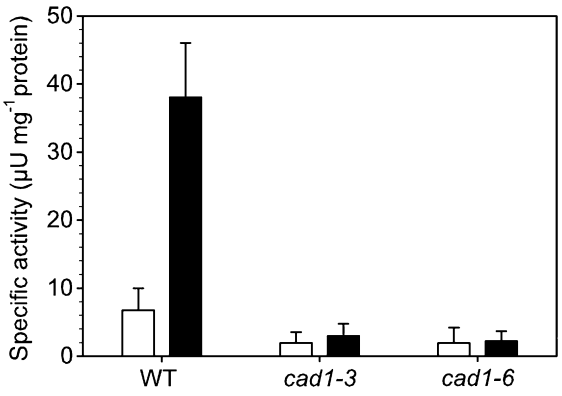

Fig. 4. Cd^{2+}-activated PCS1 specifically converts GSB in cell free extracts to γ-EC-B. Extracts of WT, *cad1-3* and *cad1-6* were incubated with 1 mM GSB in the presence of 30 µM $MgCl_2$ (white columns) or 30 µM $CdCl_2$ (black columns) for 1 h. Values shown are means ±S.D. ($n = 3$).

and the different PCS1-deficient mutants after co-infiltration with 500 µM MCB and 100 µM of different divalent metals. Samples analysed for the formation of γ-EC-B from GSB as substrate showed that in wild-type plants co-infiltration of Cd^{2+} led to a significant increase in γ-EC-B within 60 min (Fig. 5A). At the end of a 15 min labelling period the concentration of γ-EC-B formed in the presence of Cd^{2+} was already twice as high compared to control samples infiltrated with Ca^{2+} or Mg^{2+}.

The highest amount of γ-EC-B was found after co-infiltration with Cu^{2+}. In this case, the relative amount of γ-EC-B accounted for 17% of all labelled LMWTs after a 15 min labelling period and about 25% after an additional incubation period of 60 min (Fig. 5A). Co-infiltration of Ca^{2+}, Mg^{2+} or Zn^{2+} with MCB did not result in increased formation of γ-EC-B. Co-infiltration of MCB and metals with azide was compared in wild-type and *cad1-3* for Mg^{2+}, Cd^{2+} and Cu^{2+}. In all cases the amount of γ-EC-B formed was not significantly affected by azide (Fig. 5A, Table 1). In *cad1-3* and *cad1-6* no significant accumulation of γ-EC-B could be observed for any of the co-infiltrated metals (Fig. 5B and C). Addition of azide to the infiltration medium for *cad1-3* also showed now effect on the accumulation of γ-EC-B (Fig. 5B). From these observations it can be inferred that under simultaneous exposition of cells to 500 µM MCB and 100 µM heavy metals the cytosolic PCS degrades 4% of the GSB in the presence of Cd^{2+} and up to 25% in the presence of Cu^{2+}.

After formation in the cytosol GSB is rapidly transported into the vacuole by action of ATP-dependent ABC-transporters [2]. It is not known, however, whether γ-EC-B resulting from degradation of GSB in the cytosol would be a suitable substrate for vacuolar sequestration as well. To elucidate this possibility, the distribution of bimane-dependent fluorescence in leaf epidermal cells was imaged by CLSM after leaf infiltration with MCB in the presence or absence of Cd^{2+}, Cu^{2+} or the non-activating Mg^{2+}, respectively. Projections of serial optical sections taken 40 min after the incubation show complete vacuolar sequestration of the fluorescence in epidermal cells of leaves infiltrated with MCB and Mg^{2+} (Figs. 2 and 6A). When MCB was infiltrated together with Cd^{2+} or Cu^{2+} a significant amount of fluorescence was retained in the cytosol. This cytosolic fluorescence was clearly visible in several cytoplasmic

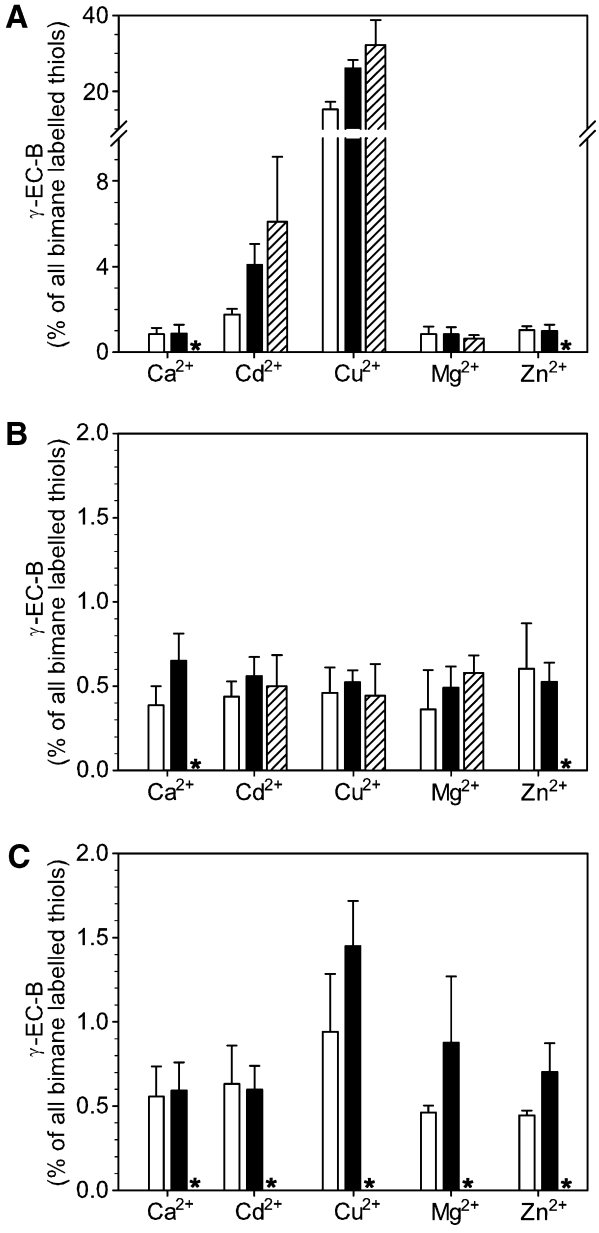

Fig. 5. Heavy metal induced activation of PCS1 leads to increased formation of γ-EC-B in intact Arabidopsis cells. Leaf pieces of WT (A), *cad1-3* (B) and *cad1-6* (C) plants were co-infiltrated with 500 μM MCB and 100 μM of chloride salts of various divalent metals, and 1 mM azide, respectively, for 15 min. Samples were taken immediately after the infiltration (white columns) or after 60 min incubation in MCB-free buffer (black columns). Samples co-infiltrated with azide were further incubated for 60 min in MCB-free buffer with 1 mM azide (striped columns). Values shown are means ±S.D. ($n = 3$). (*) not measured.

strands through the vacuole and especially in areas where the cytoplasmic strands meet the outermost cytoplasmic layer of the cell (Fig. 6B and C). This was consistent with cytosolic formation of γ-EC-B through heavy metal activated PCS1 and suggests no or only very slow sequestration of γ-EC-B to the vacuole. Infiltration of *cad1-3* leaves with MCB and Cd^{2+} in contrast did result in rapid sequestration of the conjugated bimane and thus lack of fluorescence in the cytosol 40 min after the infiltration (Fig. 6D).

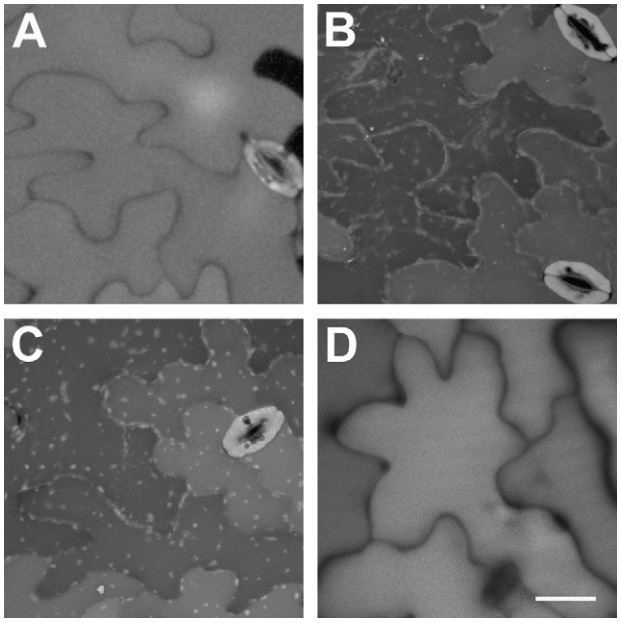

Fig. 6. Cd^{2+} and Cu^{2+} partially inhibit vacuolar sequestration of bimane-conjugates in Arabidopsis leaves. Small pieces of wild-type leaves were co-infiltrated with 500 μM MCB and 100 μM $MgCl_2$ (A), $CdCl_2$ (B), or $CuCl_2$ (C), respectively. Infiltration for 15 min was followed by further incubation in buffer without MCB and heavy metals for 40–50 min. Vacuolar sequestration of GSB was not affected by Cd^{2+} in the *cad1-3* mutant (D). All images represent projections of serial optical images collected by CLSM. The projections show the fluorescence of bimane-labelled low molecular weight thiols. Fluorescence retained in the cytosol is clearly visible by intensely labelled cytoplasmic strands. Scale bar = 20 μm.

4. Discussion

Glutathione conjugation is important in the metabolism of a broad range of herbicides. Formation of fluorescent GSB in the cytosol and rapid sequestration of this conjugate has been shown in situ [10]. From a large number of studies on pesticide and herbicide metabolism in plants it is also known that the glutathione conjugates are not end-products of the detoxification, but rather undergo further catabolism leading to formation of cysteine-conjugates [18].

The results of the experiments described here show that electrophilic xenobiotics are rapidly conjugated to GSH and sequestered to the vacuole. When the sequestration process and conjugate degradation were compared, accumulation of the respective cysteine conjugate was rather slow and required more than 48 hours to complete. In the presence of heavy metals the cytosolic enzyme PCS1 can metabolize GSB resulting in formation of γ-EC-B in the cytosol [8]. This process, however, is very slow compared with the vacuolar sequestration of the GSH-conjugate.

According to the suggestion by Beck et al. [8] PCS1 catalyses the removal of the carboxyterminal glycine residue as the first step of GSH-conjugate metabolism. Mechanistically such a degradation would represent a transpeptidation of *S*-glutamylcysteinyl conjugates onto the acceptor molecule water. *AtPCS1* is constitutively expressed, but the PCS activity of the protein is regulated by exposure to heavy metals [19,20]. Activation of the enzyme has been shown to be induced in different organisms by a wide range of metal ions, including

Cd^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+} [9]. The activation of heavy metal induced PC synthesis activity is, however, not fully understood yet. Vatamaniuk et al. [20] suggested that Cd^{2+}-binding to the protein on its own is insufficient for activation and that Cd^{2+}-GSH complexes are both substrate and activator for PC biosynthesis. In contrast, Oven et al. [21] observed that *S*-methyl-GSH, which is unable to bind Cd^{2+}, activated PCS1 only to a limited extent and that this activation was completely inhibited by Cd^{2+}. This difference might be explained by the need of reducing conditions for the PCS to achieve full activity [21].

The *cad1-3* mutant of Arabidopsis does not produce phytochelatins even after prolonged exposure to Cd^{2+} due to a point mutation in the gene encoding for PCS1 [14,19]. Based on *cad1-3* and *cad1-6*, which both lack functional PCS1, the data reported here demonstrate that PCS1 indeed has a carboxypeptidase activity in vivo, provided heavy metal ions are present at sufficient concentrations. Lack of activity of PCS2 towards GSH-conjugates in vitro might be explained by the fact that this enzyme is present in cells at very low levels and that despite the fact that it is a PCS the activity is too low to enable *cad1-3* mutants to produce any detectable PCs [14].

Under conditions of sudden exposure of plants to toxic compounds, like during herbicide spraying, it might be possible that the concentration of GSH-conjugates in the cytosol very quickly rises to rather high concentrations which are ultimately limited by the amount of GSH available. The GSH concentration in the cytosol has been reported to be in the low millimolar range [10]. With such high concentrations it may be not surprising that enzymes normally not involved in conjugate degradation use GSH-conjugates as artificial substrates. With 1 mM GSB as a substrate PCS1 can hydrolyse the conjugate in vitro [8]. This notion is also supported by the observation that free GSH can inhibit the hydrolysis of GSB in cell free extracts or with isolated PCS1 [8,22]. The hydrolysis, however, is only efficient if activating metal ions are present at sufficient concentrations as well. Moreover, it has been shown that the activity of ABC-transporters on the tonoplast is sufficiently high to remove GSB from the cytosol very quickly and that even under conditions of high exposure to bimane the concentration of GSB in the cytosol peaks between 5 and 10 min after exposure and then declines very quickly [13]. In the absence of PCS activating metals like Cd^{2+} or Cu^{2+} less than 1% of the GSB was degraded by PCS. Additional exposure to certain heavy metals might affect the kinetics for the vacuolar sequestration of GSH-conjugates in different ways. First of all, it might be possible that heavy metals directly interfere with the ABC transporters. This, however, is not the case as even in the presence of Cd^{2+} or Cu^{2+} most of the GSB was quickly sequestered. The second, more likely, possibility is that the presence of heavy metals induces the hydrolysis activity towards GSB leading to formation of γ-EC-B in the cytosol. For Cd^{2+} and Cu^{2+} it has been shown that between 4% and 25% of the GSB formed are degraded to γ-EC-B by PCS. At least partial retention of bimane-dependent fluorescence in the cytosol after infiltration of leaves with MCB together with heavy metals suggests that γ-EC-B formed under these conditions is not recognised as a suitable substrate by the ABC-transporters of the tonoplast. Retention of γ-EC-B in the cytosol is thus not surprising, especially in a situation where there are also GSH-conjugates to be transported. Given that a combination of sudden exposure to electrophilic xenobiotics and heavy metals is rather unlikely to occur in nature it is concluded that PCS1 is not the enzyme relevant for cleaving the glycine moiety from GSH-conjugates.

Glutathione-conjugates need to be sequestered to the vacuole because they would otherwise act as inhibitors for a range of different enzymes including glutathione reductase and GSTs [23]. In the past evidence for both, cleavage of glycine and glutamate as the initial step has been provided for different plant species [24]. The fast and complete sequestration of GSH-conjugates clearly indicates the first degradation step is catalysed by a vacuolar enzyme rather than cytosolic PCS1. The current data on vacuolar degradation do still not allow to draw conclusions about the initial degradation step in Arabidopsis as only Cys-B, but no intermediates accumulate. However, from the lack of degradation intermediates it can be concluded that the first degradation step is rate-limiting for the overall degradation. The presented data clearly show that degradation of GSH-conjugates in Arabidopsis takes place within the vacuole. In the absence of significant accumulation of intermediates, however, it still remains unclear how the degradation is initiated or whether even two degradation pathways starting from both termini of the GSH-moiety might work in parallel.

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